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13. ABSTRACT The regulation of the pS2 gene in MCF-7 human breast cancer cells is being examined to delineate changes that occur at the molecular level in the transformation of normal mammary cells into breast cancer cells. Although the pS2 gene is expressed in breast cancer cells and is subject to regulation by estrogens, it is not expressed in normal mammary cells. By coupling polymerase chain reaction amplification with *in vivo* footprinting, it is now possible for the first time to examine an endogenous, single copy estrogen-responsive gene residing in normally phased nucleosomes. We will (*i*) determine if the pS2 estrogen response element is occupied in the absence of hormone, (*ii*) delineate conditions needed for assembly of the basal transcription apparatus, (*iii*) identify regions of the pS2 gene involved in estrogen induced transcription activation, and (*iv*) determine regions of the pS2 gene responsible for mediating the effects of the antiestrogens, trans-hydroxytamoxifen and ICI 164,384. These investigations will enhance our understanding of how hormone-responsive genes are regulated *in vivo*, identify changes that occur at the molecular level in the progression of mammary cell development to the malignant state, and define how clinically important antiestrogens modulate transcription of estrogen-responsive genes.

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INTRODUCTION

Nature of the problem

We are interested in examining changes that occur at the molecular level in the progression of normal mammary cell development to a malignant state by studying the regulation of the pS2 gene in MCF-7 human breast cancer cells using polymerase chain reaction amplification coupled with *in vivo* footprinting. The pS2 gene is expressed in malignant, but not in normal mammary epithelium and is subject to regulation by estrogens. This gene contains the elements of a classic hormone-responsive gene: an estrogen response element (ERE), a TATA box, and a CAAT box. We will be able to examine for the first time the interaction of cellular proteins with a single copy, estrogen-responsive gene in normally phased nucleosomes. These studies will enhance our understanding of how estrogen-responsive genes are regulated in a physiological setting and provide clues as to why the pS2 gene is expressed in malignant, but not in normal mammary epithelium.

One facet of this study which has clinical implications is the examination of the effects of two antiestrogens, trans-hydroxytamoxifen and ICI 182,780, on the regulation of the pS2 gene. Tamoxifen has been used in breast cancer therapy in women with estrogen receptor (ER) positive tumors (1,2) and has resulted in a 40% reduction in breast cancer recurrence (3) as well as favorable effects on lipid profiles and bone mineral density (4,5). The importance of this drug is evidenced in a newly initiated clinical trial involving 16,000 women to test tamoxifen's ability to prevent breast cancer in women who are at high risk of developing this disease (6). With such widespread use of this drug, it seems imperative to examine in detail the effects of this drug at the molecular level. Clinical trials have also been instituted to test the ability of ICI 182,780 to prevent breast cancer recurrence (7). Genomic footprinting of this estrogen-regulated gene in human breast cancer cells should provide us with novel insights into how these drugs act at the level of the gene.

Background

a. The pS2 Gene Although the pS2 gene is expressed in many human breast cancer cells including MCF-7 cells, it is not expressed in normal mammary cells. The function of the pS2 protein is not known, but it is thought that it may be involved in growth regulation of tumor cells (8). In fact, levels of this protein have been used as a marker of estrogen responsiveness in ER-containing breast cancer cells (8-10) and an indicator of disease progression (2,11). Exposure of MCF-7 cells to 17β-estradiol activates transcription of the pS2 gene resulting in increased levels of pS2 mRNA and secreted protein (8,11-14). Promoter regions which appear to be involved in transcription activation (TATA and CAAT boxes) and estrogen responsiveness (an imperfect ERE) have been identified and verified as regions involved in hormonal regulation of this gene in transfection assays (11, 14-16).

The role of steroid hormone receptors in gene activation is poorly understood. Much of the work exploring the interaction of receptors with their cognate response elements has been carried out using synthetic promoters in transfection studies. These studies, however, involve multicopy plasmids that are not phased into nucleosomes like endogenous genes. In order to

understand how genes are regulated in target cells *in vivo*, it is now feasible to examine single copy, endogenous genes present in naturally phased nucleosomes using ligation mediated genomic footprinting. The genomic footprinting protocols, described in an original manuscript by Mueller and Wold (17) and in *Current Protocols in Molecular Biology* (18), have been used to successfully examine regulation of other inducible genes (19,20).

b. Previous experience pertinent to this proposal It has become clear that binding of the estrogen receptor to an ERE is responsible for activating transcription of estrogen-responsive genes. Therefore, I have spent the last several years examining the interaction of this receptor with DNA. The majority of my postdoctoral training was aimed at delineating mechanisms involved in ER-induced gene activation. I expressed and purified the DNA binding domain (DBD) of the estrogen receptor, characterized its effect on target cells in transient transfection assays (21), and determined that it is capable of bending ERE-containing DNA fragments in *in vitro* assays (22). I subsequently demonstrated that the intact ER present in MCF-7 human breast cancer cells was also capable of bending ERE-containing DNA fragments and that the bend induced by the intact receptor (23) was greater than the bend induced by the DBD (22, 23). Although the degree of bending observed with the unoccupied and occupied receptor is the same (23), we have recently demonstrated that an ER-induced DNA bend can be replaced by an intrinsically bent DNA sequence and effectively activate transcription (24). This indicates that DNA bending is probably an important component of transcription modulation. A large part of these investigations relied on *in vitro* gel retardation assays. Now, in order to more clearly delineate how estrogen-responsive genes are turned on in target cells, we will turn to an *in vivo* assay.

Purpose of the present work

We are interested in determining how estrogen-responsive genes are regulated in human breast cancer cells. Although transfection experiments have provided us with a great deal of information about how these genes are regulated, this type of assay exhibits significant limitations. (*i*) Cells used in transfection assays may not contain accessory proteins needed for proper regulation and if present, cells may contain these proteins in limited quantities. (*ii*) Multicopy reporter plasmids often contain synthetic promoters and sequences which bear little resemblance to native genes. (*iii*) The DNA conformation of reporter plasmids is not restricted to the nucleosomal array that would normally be found in target cells.

In order to study gene regulation in a more physiological state, it has recently become possible to examine the promoter region of endogenous, single copy genes in intact cells and to define regions of the promoter that are important in interacting with regulatory proteins. To carry out these experiments, *in vivo* footprinting is combined with polymerase chain reaction amplification (17,18). These techniques have demonstrated that regions which appear to be important in regulation from *in vitro* studies are not always the same as those determined to be important from *in vivo* studies (17,19). In addition, there are certain questions which an *in vivo* approach can address that cannot be addressed using transfection assays. Certainly one of these questions involves the DNA conformation in the area of the gene being examined. Using an *in vivo* approach, it is now possible to examine the interaction of proteins with phased nucleosomal

DNA. There has also been some question about whether the ER is associated with an ERE in the absence of hormone and whether the receptor must be continually associated with an ERE for transcription activation to be maintained. These are all questions which have been difficult to determine using currently applied techniques, but would be possible to examine using genomic footprinting. By examining the DNA regions protected in target cells when the natural DNA topology has been preserved, we should gain a more accurate understanding of the mechanisms involved in steroid hormone-regulated gene expression.

Methods

We are utilizing *in vivo* footprinting coupled with polymerase chain reaction amplification to identify cis elements responsible for mediating hormone-induced changes in transcription of the pS2 gene. Our goal is to examine this hormone-responsive gene interacting with proteins in an unperturbed system so that the promoter is allowed to maintain its "natural" DNA topology.

a. Model system used The human breast cancer cell line MCF-7 is derived from a metastatic breast cancer pleural effusion (25) and is being used in these studies to identify important cis elements involved in regulation of an endogenous, single copy estrogen-responsive gene. These cells maintain substantial levels of ER (26) and their responsiveness to estrogen has been well documented (13,14,27,28). I have previously used these cells to examine the effect of estrogen treatment on PR levels, plasminogen activator activity, cell proliferation, and ER induced-DNA bending (23,24,27-29).

Because of the comparative nature of this study, it is essential to have low basal gene expression of the pS2 gene so that when cells are exposed to estrogen, differences in the *in vivo* footprints will be evident. This will be aided by the fact that MCF-7 cells represent a homogeneous cell population. Homogeneous cell populations are recommended for genomic footprinting so that all cells respond similarly to hormone treatment resulting in footprints with a more favorable signal to noise ratio (17,18).

b. Overview of genomic footprinting The ligation mediated *in vivo* footprinting method of Mueller and Wold (17,18) has been used to identify cis elements involved in regulation of inducible genes (19,20). The advantage of this procedure is that it requires as few as 100,000 cells per reaction and allows inspection of genomic sequences involved in transcription regulation. This method will allow us to delineate regions of the pS2 gene that are associated with proteins using differential methylation of guanine residues. Upon exposure to dimethylsulfate (DMS), guanine residues from DNA regions occupied by protein are protected from methylation, while guanine residues from unprotected DNA regions are methylated. After isolation of the DNA, cleavage of methylated guanine residues with piperidine, and denaturation of the DNA fragments with heat, an oligo is annealed to a defined DNA sequence 50-90 basepairs from the region of interest. This oligo initiates synthesis of blunt-ended, double-stranded DNA fragments which originate at a defined sequence and extend to the sites of piperidine cleavage. Two complementary annealed oligos, with one blunt end and one staggered end (to prevent multimer formation), are ligated to each of the newly formed double-stranded fragments. These DNA fragments now have two defined ends and can be amplified using the polymerase chain reaction.

However, rather than using the first oligo in the amplification step, a second oligo, which anneals slightly upstream from the first oligo, is used to provide greater specificity. A third ^{32}P-labeled oligo is used to detect the amplified DNA fragments using 3-9 rounds of amplification to increase signal strength (17,18). After heat denaturation, the ^{32}P-labeled single-stranded DNA fragments of discrete lengths are fractionated on an acrylamide gel and visualized using autoradiography. A phosphorimager can also be used to quantitate the relative amount of ^{32}P-labeled DNA present in individual bands. Control DNA samples include equivalently processed genomic DNA from cells which have not been exposed to hormone, equivalently processed protein-free genomic DNA, and protein-free genomic DNA cleaved at purines or pyrimidines to serve as references for the hormone treated samples.

c. Oligos used to examine the TATA and CAAT sequences The primers LMPCR.1 and LMPCR.2 (Fig. 1A) designed by Mueller and Wold (17,18) were synthesized without modification. The linker-primer, LMPCR.1 is 25 bases long with 60% G/C content. To form the blunt-ended linker for ligation, the 25-mer is annealed to LMPCR.2, which is complementary to 11 bases at the 3' end of LMPCR.1. LMPCR.1 ligates to the 5' end of the blunt-ended, cleaved DNA, while the 11-mer, LMPCR.2, which lacks the 5' phosphate does not become covalently attached. After the ligation of these annealed oligos, LMPCR.1 is utilized to initiate synthesis of the coding strand and define the point at which cleavage of a methylated guanine residue occurs.

Three primers specific to the 5' untranslated region and first exon of the pS2 gene were designed to initiate synthesis of the non-coding DNA strand (Fig 1B). These primers are used in combination to examine the region around the TATA and CAAT sequences. The first primer, designated TATA.1, is a 20-mer with 60% G/C content and has a calculated melting temperature (T*m*) of 60°C. TATA.1 anneals to the pS2 gene from +55 to +75 and initiates synthesis of the non-coding DNA strand. First strand synthesis terminates at the sites of piperidine cleavage creating a population of double-stranded DNA fragments. The annealed blunt-ended linker comprised of annealed LMPCR.1 and LMPCR.2 is then ligated to each of the double-stranded fragments synthesized. The second primer, TATA.2, is positioned 3' of TATA.1 without overlapping sequence and extends from +27 to +51. TATA.2 is the same length and and has the same percent G/C content as the linker-primer LMPCR.1. LMPCR.1 and TATA.2 are used to amplify the population of the double-stranded DNA fragments containing sequences complementary to LMPCR.1 and TATA.2. The only double-stranded DNA fragments containing both of these sequences are those that were formed during the first strand synthesis and ligated to the blunt-ended linker. The third primer, TATA.3, is a 26-mer with 65% G/ C content and a calculated T*m* of 68°. TATA.3 overlaps with most of TATA.2 and extends three bases further 3' from +24 to +49. TATA.3 is end-labeled with $\gamma[^{32}P]$dATP and T4 DNA kinase and is then used to detect the footprint ladder. Oligonucleotides were synthesized at the Genetic Engineering Facility at the University of Illinois at Urbana-Champaign and purified using OPC columns (Applied Biosystems) or further purified as indicated.

BODY

Initial footprinting experiments DNA isolated from MCF-7 cells was treated with DMS *in vitro* for 0-60 seconds. TATA.1 was used to prime synthesis of the non-coding DNA strand. LMPCR.1 was annealed to LMPCR.2 (Fig. 1A) and the annealed primers were ligated to the newly synthesized, double-stranded DNA fragments. The DNA fragments were amplified with TATA.2 and LMPCR.1. ^{32}P-labeled TATA.3 was used to prime synthesis of a new non-coding DNA strand and the ^{32}P-labeled DNA fragments were fractionated on an 8% denaturing acrylamide gel. The ^{32}P-labeled TATA.3 migrated as discrete, major band (Fig. 2, arrow). However, there were no significant differences in the distribution or intensity of bands before or after *in vitro* exposure of MCF-7 DNA to DMS treatment, except at 60 seconds. The minor bands observed probably represented partial extensions of TATA.1 (bands above the arrow) and ^{32}P-labeled TATA.3 oligos that were not full length (bands below the arrow). Thus, our first attempts at carrying out this complex, multi-step procedure did not extend into the region of interest. Since each step in the footprinting procedure builds on the previous step, failure of any one process can result in failure of the entire footprinting procedure. Therefore, we have gone back to determine if each individual step in the overall process was working. The steps examined are listed below.

Evaluation of oligos It is extremely important that the oligos used for amplification and labeling are full length. Any variation in oligo length can result in production of spurious footprints. In fact, truncated forms of each oligo were visible when fractionated on an acrylamide gel and stained with methylene blue (Data not shown). Therefore, the oligos used to define the 5' (LMPCR.1) and 3' (TATA.3) ends of the DNA fragments were fractionated on a denaturing acrylamide gel and isolated to ensure that only the full-length species was present.

Evaluation of first strand synthesis The first step in the *in vivo* footprinting procedure involves the synthesis of non-coding DNA strands that extend from TATA.1 to the sites of piperidine cleavage. To test whether this procedure was working effectively, TATA.1 was combined with 3 μg of *in vivo* methylated, piperidine-cleaved MCF-7 template DNA in the presence of α[^{32}P]dATP and α[^{32}P]dGTP and Vent polymerase. TATA.1 successfully initiated synthesis of the non-coding DNA strands (Fig. 3). Because there was some concern that the buffer conditions might not be optimal, both the First Strand Synthesis Buffer described by Mueller and Wold (17,18) and the Vent polymerase buffer supplied by the manufacturer were tested. Both buffers promoted incorporation of the radioactive isotopes into the newly synthesized DNA strands In the absence of DNA, no product was formed (Data not shown). Thus, the first step required for *in vivo* footprinting was successful.

Evaluation of linker ligation. The second step required for *in vivo* footprinting is ligation of a double-stranded linker to the blunt-ended, double-stranded DNA fragments produced during the first strand synthesis step. We were interested in testing the ligation, but did not want to have to rely on the first strand synthesis to produce the blunt-ended DNA fragments that would serve as substrate. To test the ligation procedure alone and avoid ambiguity in determining whether the

ligation was successful, we utilized MCF-7 DNA that had been cut with RsaI to create blunt-ended DNA fragments (Fig. 1B) as the substrate for the ligation procedure.

LMPCR.1 was phosphorylated with $\gamma[^{32}P]$dATP using T4 polynucleotide kinase, unincorporated nucleotides were removed, and the ^{32}P-labeled LMPCR.1 was annealed to LMPCR.2. The annealed oligos were ligated to 3μg RsaI-cut MCF-7 DNA and fractionated on a denaturing acrylamide gel. When the ^{32}P-labeled linker was incubated with the RsaI-cut DNA and T4 DNA ligase, the linker was successfully ligated to the DNA fragments as demonstrated by the decreased mobility of the ligated DNA fragments (Fig. 4, large arrow). However, when no DNA was included in the reaction, only the ^{32}P-labeled oligo was visible (small arrow). In the *in vivo* footprinting procedure outlined by Mueller and Wold (17,18), ligation of the linker is carried out directly after the first strand synthesis without removing the buffer used during the first step. Because there was some concern that this buffer system might not be optimal for ligation, we tested the Gibco-BRL ligase buffer alone (GB) and in combination with either the First Strand Synthesis buffer (1SS) or the Vent polymerase buffer (Vent) supplied by the manufacturer. All three buffer systems appeared to work equally well in promoting the ligation of the ^{32}P-labeled linker. Thus, we have demonstrated that the second step required in the footprinting process, ligation of the linker to double-stranded DNA, was successful.

Evaluation of annealing and extension temperatures used in amplification The banding pattern we had observed with our initial footprinting experiments appeared to indicate that no productive amplification was taking place (Fig. 2). Therefore, we tested the TATA primers using conventional PCR to determine whether they could be successfully used to prime polymerization of the non-coding DNA strand. Another gene-specific primer was needed to prime synthesis of the coding strand in standard, bidirectional PCR. A new primer, pS2 5', was synthesized with the same number of nucleotides (25 bases) and the same predicted T*m* as TATA.2. This primer anneals to the pS2 gene from -666 to -642 (Fig. 1B). By utilizing the pS2 5' oligo instead of LMPCR.1, we eliminated the first strand synthesis and ligation steps and were able to directly assess the efficiency of the amplification process alone. No product was observed using 2 μg of Eco RI-cut genomic DNA template when the buffer described by Mueller and Wold (17,18) was utilized (Data not shown). However, when the reactions were carried out in the Vent buffer supplied by the manufacturer, appropriately sized DNA fragments were observed (Fig. 5). The double-stranded DNA fragments were, respectively, 740, 717, and 715 basepairs in length when TATA.1, TATA.2, and TATA.3 were used as 3' primers. To optimize the procedure, different annealing temperatures were tested as indicated. Extension temperatures of 74° (Lanes 1-6, 8-11) or 75° (Lanes 12,13,15) were also tested. Annealing temperatures of 62° for TATA.1, 70° for TATA.2, and 72° for TATA.3 appeared to work well. Higher annealing and/or extension temperatures clearly had detrimental effects on DNA amplification (Lanes 6,13). Since contamination can be problem when many PCR cycles are incorporated in a procedure, we also wanted to be sure that the products we observed were dependent upon the presence of the MCF-7 template. As expected, when no DNA was added to the reaction, no product was present (Lanes 1,3,5,8,10,12). The calculated and empirically determined melting temperatures for each of the primers are listed in Table 1.

Evaluation of the overall footprinting procedure From the studies described above, we were able to demonstrate that several of the individual steps involved in the footprinting procedure were working. However, because footprinting relies on the success of each individual step, we also wanted to test these procedures in succession to ensure that the entire process would be successful. We utilized MCF-7 DNA that had been cut with Rsa I to introduce a blunt end at -317 (325 bp fromTATA.3) rather than methylated, piperidine-cleaved DNA so that the 5' end of the DNA fragment synthesized in the first step would be defined. TATA.1 was used to carry out 10 cycles of first strand synthesis at 62°. Annealed LMPCR.1 and LMPCR.2 were ligated to the double-stranded DNA fragments produced. TATA.2 was used in conjunction with LMPCR.1 to amplify the intervening DNA sequence with annealing and extension temperatures of 70° and 74°, respectively. ^{32}P- labeled TATA.3 was used to detect the amplified 392 nucleotide product (Fig. 6). A ^{32}P-labeled DNA fragment 427 basepairs in length was included on the same gel for reference. Thus, we have been able to successfully carry out almost all of the required procedures in succession and obtain the desired product. From this point we only need to include DMS treatment and piperidine cleavage to successfully carry out the entire footprinting procedure.

CONCLUSIONS

As described in the Statement of Work, Task 1 is to "Carry out initial footprinting experiments" during the first year of funding. We feel we have made substantial progress in this area. We have worked extensively over the last year to address Subtask 1a, which is to "Determine the optimal conditions for footprinting" by developing the single-step procedures required for the entire footprinting process. In addition, we have also been successful in carrying out almost all of the required steps for ligation-mediated *in vivo* footprinting in succession as indicated in Fig. 6.

We have addressed Subtask 1b, which is to "Examine TATA and CAAT boxes for interaction with proteins in the presence and absence of hormones." The TATA primers described in this report were used to successfully prime synthesis of the noncoding DNA strands. These same primers and procedures will be used to examine the TATA and CAAT boxes. Now that many of the basic procedures have been worked out, we are carrying out additional experiments with RsaI-cut MCF-7 DNA to ensure that we reproducibly obtain a ^{32}P-labeled DNA fragment of the appropriate size. Next, we will use MCF-7 DNA that has been treated with DMS *in vitro* and cleaved with piperidine to make sure that we can obtain reproducible footprints using all of the required steps. We will then move on to examine *in vivo* methylated DNA isolated from MCF-7 cells that have or have not been exposed to hormone. We have already isolated DNA from MCF-7 cells that were maintained in phenol red-free medium for one week, transferred to serum free medium for 3 days, exposed to either 10 nM 17β-estradiol or ethanol vehicle for 40 minutes, and then treated with 2% DMS for 2 minutes at 37°. We are also in the process of determining the optimal *in vivo* DMS exposure time required for footprinting. We have isolated piperidine-cleaved DNA from MCF-7 cells that were exposed to DMS *in vivo* for 0, 0.5, 1, 1.5, or 2 minutes. Thus, we have accumulated most of the materials and have made substantial progress mastering the procedures required to complete the work outlined in Subtasks 1a and 1b.

Subtask 1c will "Determine if the pS2 ERE is occupied in the absence of estrogen." This Subtask has not yet been addressed in detail. We have, however, determined the sequence of the three oligos that will be used to examine the region surrounding the ERE. We have purchased the oligo needed for first strand synthesis and found that the incorporation of radioactive nucleotides into the new DNA strand is similar to the incorporation observed with TATA.1. We will purchase the other two oligos required to examine the ERE when we were sure that the footprinting procedure is reproducibly successful. We have isolated DNA from MCF-7 cells that were either maintained in a hormone-free environment or treated with 10 nM 17β-estradiol for 24 hours and then exposed to DMS *in vivo* for 2 minutes. These DNA samples will be utilized to address Subtask 1c.

Ligation-mediated *in vivo* footprinting is an extraordinarily complicated procedure that requires the utmost attention to detail and careful analysis of each step to ensure success. Although it appeared at the onset that we would be able to utilize the protocol as designed by Mueller and Wold (17,18), we have found that several of the procedures required modification or a substantial investment of time to work out conditions. In retrospect, we were probably overly optimistic about the time frame required to work out the individual steps of this procedure. However, we have made significant progress in getting the overall procedure to work and have addressed Subtasks 1a, 1b and 1c during this first year of funding.

If we have difficulty reproducibly detecting footprints after *in vivo* DMS treatment, we may use DNase I treatment of MCF-7 nuclei rather than DMS treatment of cells in culture. This would eliminate the use of piperidine cleavage and the problems associated with inhibition of enzyme activity caused by incomplete removal of this substance and would include all of the procedures that we have already carried out successfully. After determining that the footprinting methodology is successful with DNase treated nuclei, however, we would probably return to DMS treatment since the resolution of protein-DNA interactions is substantially better and perturbation of cells is significantly less with this method. We are currently carrying out *in vitro* DNase I footprinting analysis of the pS2 gene in our laboratory and it would be very informative to compare the *in vitro* and *in vivo* footprints.

During the coming year we hope to complete Subtasks 1a, 1b, and 1c. We will also examine Task 2, which is to "Determine the effects of antiestrogens using *in vivo* footprinting." The oligos used in Subtasks 1b and 1c will also be utilized to exmaine the TATA and CAAT boxes and the ERE from MCF-7 cells that have been treated with 4-hydroxytamoxifen or ICI182,780. Since the technical difficulties involved in working out the footprinting procedure should be solved by the time we initiate these studies, we should be able to make progress rapidly and complete these experiments in the coming year. Comparison of footprints from cells that have been maintained in a hormone-free environment or exposed to estrogen or antiestrogen should be very informative and provide insight into how estrogens and antiestrogens modulate gene expression.

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FIGURE LEGENDS

Figure 1. Sequence of primers and the pS2 5' gene used in *in vivo* footprinting. Panel A. The sequences of the linker/primer oligo LMPCR.1 and the linker oligo LMPCR.2 as designed by Mueller and Wold (17,18) are shown. Panel B. The pS2 gene sequence from -703 to +130 is shown (11,15). The position of the transcription start site (+1), the TATA and CAAT boxes, and the primers pS25', TATA.1, TATA.2, and TATA.3 are indicated. The coding sequence for the pS2 protein begins at +41.

Figure 2. Initial footprinting experiment. MCF-7 DNA (2μg) was exposed to DMS *in vitro* for 0-60 seconds, isolated, and then used as a template for first strand synthesis with TATA.1 as the primer. The newly synthesized double-stranded DNA fragments were ligated to annealed LMPCR.1 and LMPCR.2. The ligated, double-stranded fragments were amplified using TATA.2 and LMPCR.1. The resulting DNA fragments were labeled with ^{32}P-labeled TATA.3. The ^{32}P-labeled DNA fragments were fractionated on a denaturing 8% acrylamide gel. The gel was dried and subjected to autoradiography. The arrow indicates the position of the ^{32}P-labeled TATA.3 primer.

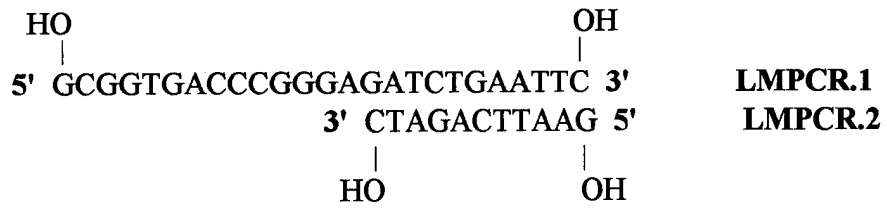
Figure 3. First strand synthesis using TATA.1 as a primer. MCF-7 DNA, which had been exposed to DMS for 2 minutes *in vivo*, was incubated with TATA.1 in the presence of [^{32}P]dATP, [^{32}P]dGTP, and either First Strand Synthesis Buffer (17,18) or Vent polymerase buffer. Vent polymerase was added and the reaction was subjected to 3 rounds of first strand synthesis. 2.5 μl of the 30 μl reaction was fractionated on an 8% denaturing acrylamide gel. The gel was dried and subjected to autoradiography.

Figure 4. Ligation of the linker and to RsaI-cut MCF-7 DNA. ^{32}P-labeled LMPCR.1 was annealed to LMPCR.2 and then incubated in the absence (-) or in the presence (+) of 3 μg RsaI-cut MCF-7 DNA. Buffers utilized in the reaction were the Gibco BRL ligase buffer supplied the manufacturer alone (GB) or in combination with First Strand Synthesis Buffer (1SS, Ref. 17,18) or the Vent polymerase buffer (Vent) supplied by the manufacturer. The ^{32}P-labeled DNA fragments were fractionated on a denaturing 8% acrylamide gel. The gel was dried and subjected to autoradiography. The positions of the ^{32}P-labeled linker alone (small arrow) and DNA ligated to the linker (large arrow) are indicated to the right of the figure.

Figure 5. Optimization of annealing and extension temperatures for amplification. The pS2 5' oligo (Fig. 1) was incubated with either TATA.1, TATA.2, or TATA.3 in the absence (-) or presence (+) of 2 μg MCF-7 DNA in order to amplify the pS2 gene sequence. The annealing temperatures are indicated. Extension temperatures of 74° (Lanes 1-6, 8-11) or 75° (Lanes 12, 13, 15) were utilized. The amplification reactions were fractionated on a 1% agarose gel and stained with ethidium bromide to visualize the bands. The 740, 717, and 715 baspair DNA fragments synthesized with TATA.1, TATA.2, and TATA.3, respectively, are clearly visible. Gibco BRL 1 kb molecular weight markers (M) were included on the gel.

Figure 6. First strand synthesis, ligation, amplification , and labeling of the DNA product. TATA.1 was used to prime synthesis of the non-coding strand of RsaI-cut DNA. The double-stranded DNA products were ligated to the annealed oligos LMPCR.1 and LMPCR.2. LMPCR.1 and TATA.2 were used to amplify the double-stranded DNA fragment using 70° annealing and 74° extension temperatures. The DNA fragments were subjected to three rounds of amplification using ^{32}P-labeled TATA.3 as a primer. The final products were fractionated on an 8% denaturing acrylamide gel and subjected to autoradiography. The ^{32}P-labeled 391nucleotide product is indicated with an arrow. A 427 basepair ^{32}P-labeled DNA fragment was included on the same gel as a size marker (M).

A



B **pS2 gene sequence**

-701 -681 -661 *pS25' primer*
AAG TGATTCTCCT GACTTAACCT CCAGAGTAGC TAGGATTACA GGCACCCGCA
-641 -621 -601
CCATGCCTGG CTAATTTTTG TATTTTTTTT TTTTGTAGAG ACGGGGTTTC GGCCATGTTG
-581 -561 -541
GCCAGGCTAG TCTCAAACTC CTGACTTTAA GTGATCCGCC TGCTTTGGCC TCCAAAGTGT
-521 -501 -481
TGGGATTACA GCGTGAGCCA CTGCGCCAGG CCTACAATTT CATTATTAAA ACCAATTCCA
-461 -441 -421
CTGTAAAAGA ATTAGCTTAG GCCTAGACGG AATGGGCTTC ATGAGCTCCT TCCCTTCCCC
-401 *pS2 ERE* -381 -361
CTGCAAGGTC ACGGTGGCCA CCCCGTGAGC CACTGTTGTC AGGCCAAGCC TTTTTCCGGC
-341 -321 *Rsa I* -301
CATCTCTCAC TATGAATCAC TTCTGCAGTG AGT↓ACAGTGC GGGAGGGCCT CTCAGATATG
-281 -261 -241
AGTAGGACCT GGATTAAGGT CAGGTTGGAT TTACCCTGAG GAGACTCCCA TGGGAAAGAG
-221 -201 -181
GGACTTTCTG AATCTCAGAT CCCTCAGCCA AGATGACCTC ACCACATGTC GTCTCTGTCT
-161 -141 -121
ATCAGCAAAT CCTTCCATGT AGCTTGACCA TGTCTAGGAA ACACCTTTGA TAAAAATCAG
-101 -81 *CAAT box* -61
TGGAGATTAT TGTCTCAGAG GATCCCCGGG CCTCCTTAGG CAAATGTTAT CTAACGCTCT
-41 *TATA box* -21 -1 +1
TTAAGCAAAC AGAGCCTGCC CTATAAAATC CGGGGCTCGG GCGGCCTCTC ATCCCTGACT
21 *TATA.3 primer* 41 *TATA.2 primer* 61 *TATA.1 primer*
CGGGGTCGCC TTTGGAGCAG AGAGGAGGCA ATGGCCACCA TGGAGAACAA GGTGATCTG
81 101 121
CGCCCTGGTC CTGGTGTCCA TGCTGGCCCT CGGCACCCTG GCCGAGGCCC AGACAGGTAA

Figure 1

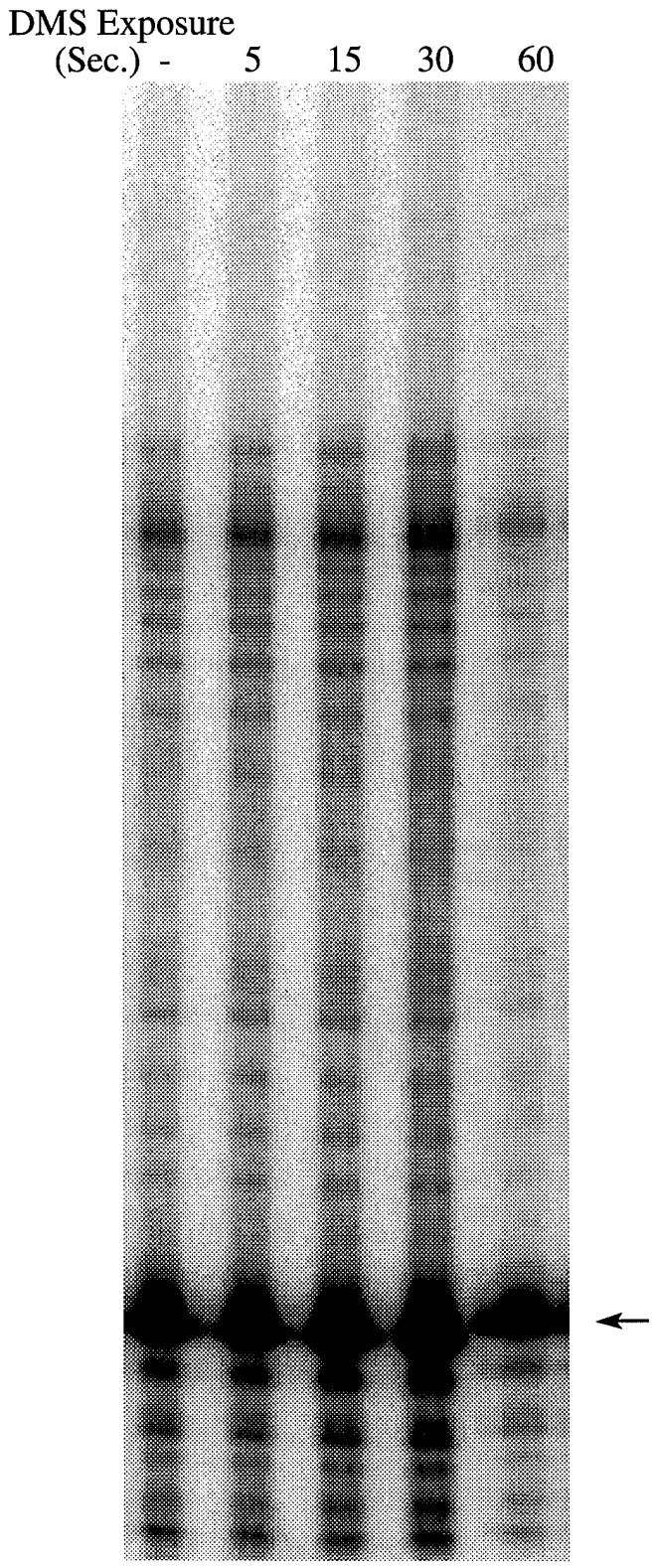


Figure 2

Buffer 1SS Vent

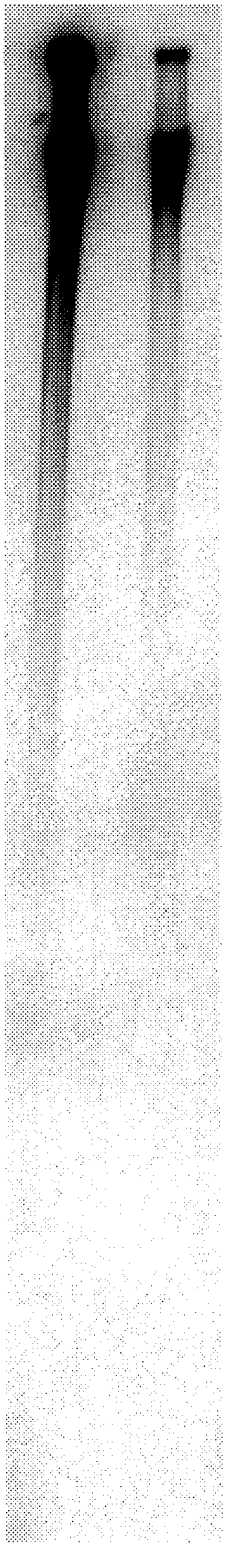


Figure 3

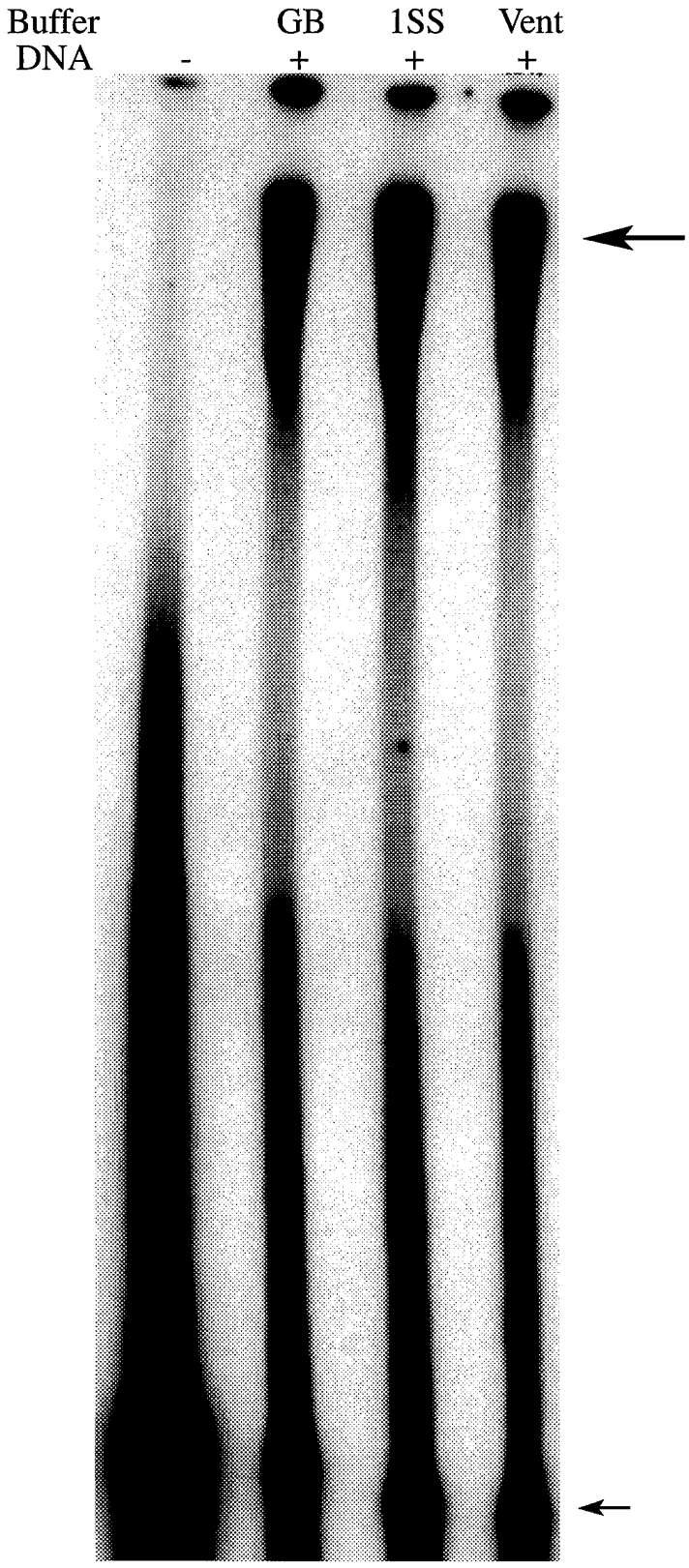


Figure 4

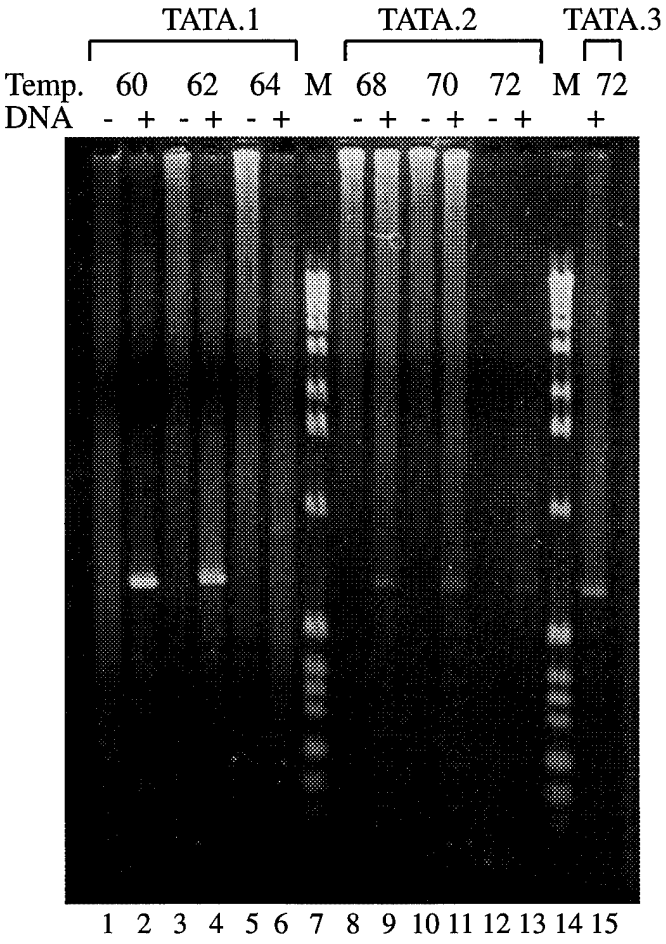

TATA.1
TATA.2
TATA.3
Temp. 60 62 64 M 68 70 72 M 72
DNA - + - + - + - + - + - + - + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 5

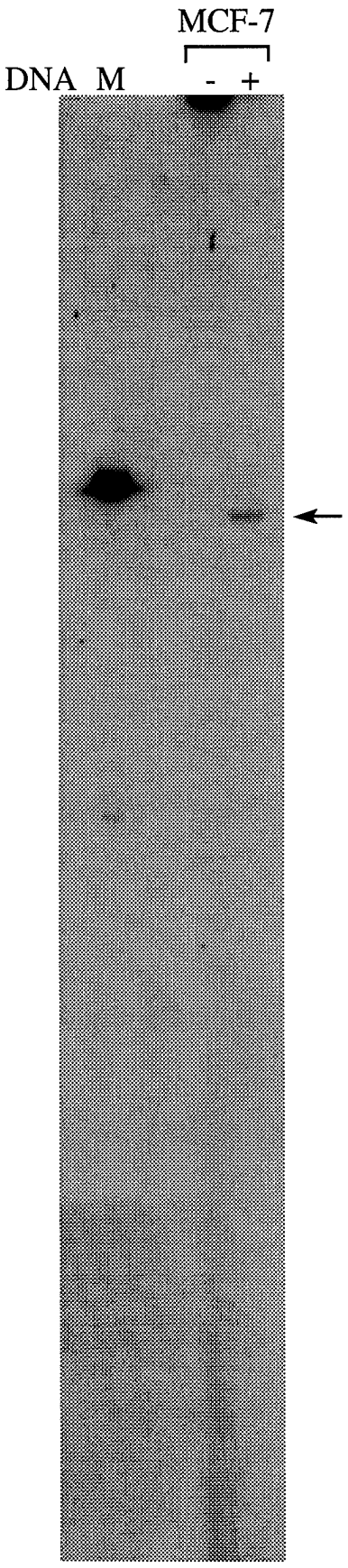


Figure 6

Table 1 primer Tm's

Method used to determine Tm	TATA.1	TATA.2	TATA.3	LMPCR.1
Calculated[1] for Current Protocols buffer	59.9	64.9	67.9	64.9
Calculated for Vent buffer	62.1	67.9	70.7	67.9
Calculated by DNA*	65.8	68.1	67.7	ND
Calculated[2] by GC content	64	80	86	80
Empirical	62	70	72	ND

[1] Tm = 81.5 + 16.6(log salt molarity) + 0.41(%GC) - (500/length). The salt concentration of the two buffers is significantly different.

[2] Tm = 4(number G or C) + 2(Number A of T)